Potential Application of Immunoassay for the Detection of Pesticide Residues in Agriculture

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ABSTRACT

In Taiwan, the Agricultural and Forestry Agency (AFA) and Food and Drug Administration (FDA) routinely monitor the pesticide residues in agricultural products; samples were collected from production districts, packing sites and all kinds of markets. Currently the multi-residue testing methods of pesticides promulgated by the Ministry of Health and Welfare can determine the residues of 373 pesticides or their metabolites. However, expenses for the installation of needed analytic instruments, labor, and other cost are too expensive for most people, besides it cannot prevent the risky shipments in time from further trade. A rapid bioassay of pesticide residues (RBPR) method targeting on the neurotoxic orgranophosphorus and carbamate insecticides was developed by Taiwan Agricultural Research Institute in 1985 using the acetylcholinesterases extracted from a susceptible housefly strain. In 2017, 360 RBPR stations inspected 1.26 million shipments and a total of 37 tons of agricultural products with toxicity risk were destroyed. Since many of these toxic insecticides were banned in the past two decades, research on developing immunoassay, aiming at 30 insecticides and fungicides that were frequently found in violated fruits and vegetables by FDA, was launched in 2011 to strengthen the RBPR stations with enhanced residue control capability. In a 4-year program, highly specific and sensitive antibodies against 18 pesticides as well as direct and indirect competitive ELISA protocols have been developed with the capability of reaching the detection limits ranging from 0.01 to 2.24 ppb. Commercial test strips and ELISA kits have been under development through technical transfer of the antibodies

and the assay protocols to biotechnology companies since 2016. The first product is a test strip that can detect the residue of dimethomorph in 10 min with the cut-off value of 1 ppm. By making adequate dilutions during the extraction process, the rapid test is able to obtain the result that meets the tolerance standard of various crops, e.g., 0.6 ppm and 2.5 ppm for onion and small leafy vegetables, respectively.

Keywords: immunoassay of pesticide residues, enzyme-linked immunosorbent assay (ELISA), colloidal-gold based lateral-flow immunoassay

INTRODUCTION

To ensure the high yield, many pesticides are mixed and frequently used during crop production to combat the damage cause by pests, diseases and weeds. Located in the sub-tropical region, the year-round cropping system in Taiwan facilitates the propagation of all pests and has accelerated the pest resistance problem; hence the farmers tend to use more pesticides to guarantee effective control.

In order to monitor and understand general situation of pesticide residues in fruits and vegetables, Taiwan has been conducting residues monitoring program since early 1960s. The earliest residue control method in Taiwan used alive housefly adults as testing organism, which was developed by Taiwan Agricultural Research Institute (TARI) during 1959-1962. This 4-hr bioassay method was implemented in 18 wholesale markets around Taiwan, and had been replaced in 1985 by the rapid bioassay for pesticide residues (RBPR) technology that takes only 10 min per assay.

The RBPR method uses the neuroenzyme, acetylcholoinesterases (AChE), extracted from the heads of susceptible housefly colony to detect the cumulative toxicity of organophosphorus and carbamate insecticides [5, 2]. The theory has been echoed by the Food Quality Protection Act (FQPA) of US EPA in late 1990s, which emphasizing the importance of cumulative toxicity of these two groups of insecticides in its report on "*The Use of Data on Cholinesterase Inhibition for Risk Assessment of Organophosphorus and Carbamate Pesticides* [12].

Currently, RBPR network supervised by TARI includes users from farmer's groups, wholesale markets, food suppliers, supermarket chain stores, fast food companies, etc. In 2017, 360 RBPR stations inspected 1.26 million shipments that resulted in demolished 37 tons agricultural products with higher residue risk before consumption. For instance, the RBPR station at Taipei Agricultural Produce Market

has been conducting inspection since 1985; they randomly checks 150-180 shipments every day during midnight hours. All testing results, including double check, need to be finished before auction starts at 3:00 AM, and any shipments with higher residue risk will be withheld on-site [3].

Chemical analysis was not prevailing before 1990s in Taiwan due to the technology and instruments were still new for most countries, and could only detect the residues of 79 pesticides in early 2000s. However, great progress has been made in the analytical methodology, reagents, apparatus, instruments, etc. in the past decade, particularly when new models of GC/MS/MS and LC/MS/MS become more efficient and the detection limits rise to ppb levels. The number of pesticides monitored by multi-pesticide analysis method has thus increased from 136 pesticides in 2006 to 373 pesticides in 2017 [7].

In 2017, the Food and Drug Administration (FDA) inspected a total of 3,341 shipments, and 363 shipments (10.9%) were confirmed to violate the food safety standards [4]. However, the cost at 200 US dollars per sample and time spent is still too high for most growers. Hence, a test method with low cost and fast screening is definitely helpful.

Due to more and more pesticides were registered and a lot of neurotoxic insecticides were banned in the past ten years, RBPR that targeting only on organophosphorus and carbamates insecticides can no longer fulfill current needs. In this regard, TARI decides to initiate the research on immunoassay method aiming at pesticides commonly detected by chemical analysis in 2011.

Development of Immunoassay Technology

The immunoassay technology is based on the principle of specific binding of antibody and antigen. By using antibody as a biosensor, techniques for qualitative and quantitative analysis of target compounds, enzymes or proteins are then developed. Currently, immunoassay methods such as radioimmunoassay (RIA), luminescent immunoassay (LIA), fluorescence immunoassay (FIA), enzyme-linked immunosorbent assay (ELISA), and colloidal-gold based lateral-flow immunoassay are widely used in medicine, plant pathology or quality control in various fields. ELISA technique was first published by Engvall and Perlman in 1971, and has been widely used as diagnostic tools. Due to the highly specific, sensitive as well as the qualitative and quantitative

features, the amount of antibody or antigen can be estimated and is very adequate to be used as rapid pesticide residue assay method. The ELISA method uses enzymes such as horseradish peroxidase (HRP) or alkaline phosphatase (AP) to label antibodies or antigens, after adding substrate of the enzyme, product obtained in the enzyme reaction can be detected by spectrophotometer and calculate the level of color change. For development of immunoassay for pesticide residues, the individual pesticide analogs (hapten) were conjugated with carrier proteins before being inoculated into animals to generate antibodies against the coupled compound from animals, for pesticide itself is too small to induce immunity of the animal.

In the past two decades, immunoassay technology becomes more and more important particularly in human medical diagnosis for its specific and sensitive characteristics, and related test eagents, apparatus and instruments with good quality are readily available. A number of ELISA methods are also derived to meet various needs, for instance, direct competitive enzyme-linked immunosorbent assay (dc-ELISA) and indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) are commonly used to detect pesticide residues.

In the dc-ELISA, pesticide antibody is immobilized on the microplate. The pesticide and pesticide hapten-enzyme conjugate are then added and will compete with each other for binding to the pesticide antibody. The pesticide hapten-enzyme conjugate refers to the pesticide hapten with labeled enzyme such as HRP or AP. After washing, the unbound pesticide hapten-enzyme conjugates are removed; only the binding complex of the pesticide hapten-enzyme conjugates with pesticide antibody is left in the microplate. Then, the substrate was added for the color reaction that were measured by ELISA reader to quantify the pesticide residues; the higher the residual pesticide concentration, the lighter the final coloration.

As to the ic-ELISA, the control antigen, an antigen prepared by linking a pesticide hapten to a protein of a non-immune antigen carrier, is adhered to the microplate first, and then adds pesticide and pesticide antibody (primary antibody). The control antigen will compete with pesticide to conjugate with the pesticide antibody. After washing and removing the free portion, only the binding complex of the control antigen and the pesticide antibody is left. Then add the enzyme-labeled secondary antibody that can recognize the primary antibody and bind with it. After washing and removing the free part, only the conjugate of control antigen, pesticide antibody and the secondary

antibody is left in the microplate. By adding the substrate, qualitative calculation of the pesticide concentration done by the color expression was recorded by the ELISA reader to quantify the pesticide residue. In the ic-ELISA, the higher the concentration of the pesticide, the lighter the final coloration.

In comparison, the procedure of ic-ELISA is more complicated because of the multiple binding of primary antibody and secondary antibody. However, the signal amplification and sensitivity are usually higher, and is commonly used in academic research. On the other hand, procedure of the dc-ELISA is simpler to the users, and is suitable for routine and large-scale testing.

Another rapid detection immunoassay method in medicine is colloidal-gold based lateral-flow immunoassay, and the competition approach is widely used to detect toxin [17] and drug residue in animal foodstuffs [1]. The mechanism of competition approach is crosslink the antibody of drug to colloidal gold (colored material) below sample pad first, after antibody-colloidal gold complex combine with the antigen (drug) in sample solution, the reference antigen in T line of two stealth lines on the test paper can not capture the antibody-colloidal gold-drug, T line will be extreme light or invisible. If there is no drug in sample solution, the reference antigen in T line will capture most of the antibody-colloidal gold complex, and T line will shows up. This technique does not require expensive equipment and easy to operate that may have great potential on pesticide residue detection.

Develop immunoassay kits for pesticide residues screening at TARI

In 2014, aiming at the high-risk pesticides that were frequently detected in the unqualified products in the market, TARI applied the immunological principle of specific binding of antigen and antibody to develop the rapid screening technology for pesticide residues. Curretly, polyclonal antibodies against 18 pesticides have been developed, which include 8 insecticides (acetamiprid, fipronil, thiamethosam, chlorfenapyr, pyriporxyfen, profenophos, deltamethrin, and bromopropylate), 9 fungicides (dimethomorph, difenoconazole, tebuconazole, chlorothalonil, pencycuron, hexaconazole, oxycarboxin, pyraclostrobin, and azoxystrobin) and 1 herbicide (2,4-D). The detection limit (10% inhibition rate of ic-ELISA) of the best antibodies obtained ranging from 0.01 to 2.24 ppb, and the cross-reactivity to 88 pesticides from 50 chemical categories is less than 0.1%.

1. Dimethomorph Rapid Test Kit

Dimethomorph antibody and the test protocol has been transferred to the Taiwan Advance Bio-Pharm., Inc. to develop dimethomorph rapid test kit in late 2016. The cut-off value of the test strip is 1.0 ppm, and the assay can be complete in 10 min. Two invisible lines are bound on the test paper, the upper part is the C line (Control-line), and the lower part is the T-line (Test-line: containing the hapten (dimethomorph)-carrier protein complex). When the concentration of dimethomorph in the sample is less than the cut-off value, 1.0 ppm, T-line will capture most of the dimethomorph antibody-colloidal gold complex and T line will shows up; while if the concentration of dimethomorph in the sample is greater than or equal to 1.0 ppm, the T line will be extreme light or invisible.

The cut-off value of the dimethomorph rapid test kit is set at 1.0 ppm. For practical purposes, the tolerance level for various crops might vary, hence adequate dilutions should be made during the extraction process (Figure 1). For example, the tolerance levels for small leafy vegetable and cabbage lettuce are 2.5 and 10.0 ppm in Taiwan, respectively [(7)]. Sample extracts are obtained by weighing 0.5 g sample first, and then add 1.25 ml and 5 ml extraction solution for leafy vegetable and cabbage lettuce, respectively. As to the grape, strawberry, tomato, cucumber and onion, the tolerance levels are 3, 2, 1.5, 1 and 0.6 ppm, respectively. Extracts are prepared by weighing 1 g of sample for individual crop, then add 3, 2, 1.5, 1 and 0.6 ml extraction solution, respectively and stir thoroughly. Then, follow the normal assay procedure to estimate the residue status.

2. ELISA kit designs for multi-pesticide assay

Some pesticides are commonly used in certain crops e.g., tomato and bell pepper, hence to develop an assay kit that can detect the residues of 4 or 8 pesticides at one time with qualitative and quantitative results might be feasible. In this regard, four pesticides are chosen to develop multi-pesticide assay kit, and they are acetamiprid, fipronil, dimethomorph and tebuconazole. Two concentrations for each pesticide were chosen that gave the inhibition rates of 20-60%, and the detection limit of the above-mentioned four pesticides can be obtained by the regression between the logarithm of the pesticide concentrations and the reaction inhibition rates; they are 0.68, 1.14, 0.23, and 0.61 ppb, respectively. Less than 0.1% cross-reactivity with other 88 non-target pesticides from 50 chemical groups indicated their specificity and sensitivity. Each ELISA plate is pre-

coated with 4 pesticides antibody, and the ELISA kit has the ability to analyze 4 pesticides concentration with 9 samples simultaneously (Figure 2).

To confirm the influence of sample matrix on ELISA, detailed study on the assay protocol of komatsuna (*Brassica rapa* var. perviridis) against these four pesticides were conducted, and the tested concentration was set at 1.0 ppm for all pesticides. Average recoveries of acetamiprid, fipronil, dimethomorph and tebuconazole from komatsuna were 74.0%, 105.3%, 92.8%, and 92.1%, respectively. The pesticide recoveries were between 60-140%, which is comparable to the current inspection method announced by the Food and Drug Administration [(9)]. The CV values were 3.3%, 3.8%, 4.1%, and 6.9%, respectively, which also indicated the stability of this analytical method. The entire operation took only 1.5 hours from sample extraction, dc-ELISA reaction, and pesticide concentration interpretation.

DISCUSSION

Pesticide immunoassay technology has been developed in many countries, and commercial products are available from Japan, the United States, Canada, Norway, etc. The HORIBA Company in Japan develops the Smart Assay series, including 21 ELISA kits for the detection of the residues of insecticides and fungicides by using direct competitive ELISA (HORIBA website). Other ELISA-based pesticide residue analysis products such as ic-ELISA or dc-ELISA kits are produced from Abraxis, EnviroLogix and Bio Scientific companies. The colloidal gold lateral flow immunoassay is a very handy method for on-site residue screening since the technique does not require expensive equipment and easy to operate; however, it requires highly sensitive antibody and is more challenge on wider detection spectrum. Although Wang *et al*. (2015) had developed chlorpyrifos colloidal gold lateral flow immunoassay and the minimum detection limit is 4.0 ppm and Hua *et. al*. (2012) screened a single antibody supplemented with colloidal gold lateral flow immunoassay to identify eight organophosphorus pesticides, their commercialized pesticide test strip is still uncommon.

There are more studies on pesticide residue analysis by ELISA. Watanabe *et al.* (2013) had compiled several reports on the thiamethoxam pesticide ELISA test. The detection limit of the ELISA is 0.05 ppb, which is even more sensitive as compared to that by the HPLC/MS/MS analysis, 0.14 ppb. After addition of thiamethoxam on

cucumbers and tomatoes, the samples quantified by ELISA only diluted with methanol, further diluted and subjected to assay. However, the samples quantified by HPLC needed to be extracted with several organic solvents and SPE first, and then concentrated and adjusted to proper concentration before HPLC analysis. The results showed that the pesticide recovery and accuracy of the two methods were equivalent.

According to the multi-residue analytic method for foods announced by the Food and Drug Administration, the procedures of sample cleanup are very complicated including the necessary use of a lot of highly toxic organic solvents and the long preparation time. After the tedious solvent extraction, purification, drying, re-dissolution and filtration processes, the sample extracts can then be injected into LC/MS/MS and GC/MS/MS [7]. In contrast, the ELISA method use a much more simple extraction process and does not require the use of highly toxic organic solvents. Watanabe *et al.* (2005) added the pesticide chlorfenapyr to apple and peach juice, extracted with methanol for 3 minutes, and the extract was filtered through a filter and diluted 25 times with buffer. The recovery of the obtained pesticide was 88.0-100.0%. Watanabe *et al*. (2007) also applied ELISA to determine residual imidacloprid in fruit juices. The recovery rate of imidacloprid in apple, grape and orange juice were 82.0-128.7%. Watanabe and Miyake (2013) added the thiamethoxam to homogenates of cucumber and tomato, and the recovery rates of thiamethoxam were 88.4-110.2%. Qian *et al*. (2009) added the pesticide chlopyrifos-methyl to cabbage and grapes, and tested it with ELISA; and the recoveries of chlopyrifos-methyl ranged from 82.4 to 94.6%. The above test results indicated that the pesticide ELISA detection method could be simple and highly efficient.

CONCLUSION

The year-round production and small-scale farming system have contributed to very complicated pesticide residue problems in Taiwan. The R&D of immunoassay methods for pesticides with higher residue risk and the commercialization of rapid test kit can provide an effective tool for growers who are willing to understand the residue status of the fruit and vegetable they produced before harvest. In the 4-year research program, antibodies of 18 pesticides are successfully achieved with detection limits ranging from 0.01 to 2.24 ppb, and the run time to complete an assay for test strip and ELISA kit are 10 min and 1.5 hours, respectively. Standard sampling and assay

procedure for more than 30 crop species are also established. Currently, local biotechnology companies have already transferred specific antibodies and the test protocol of individual pesticide to produce commercial kits, and the potential users might include consumers, farmers or the RBPR stations in the production districts, wholesale markets, supermarket chain stores, etc. In the future, test strip and ELISA kit for testing one pesticide, or an ELISA kit designed for the detection of 4 or 8 pesticides simultaneously will be possible, with the hope that consumers can have more confidence on the safety of harvested agricultural products.

LITERATURE CITED

1. Byzova, N. A., Smirnova, N. I., Zherdev, A. V., Eremin, S. A., Shanin, I. A., Lei, H. T., Sun, Y., and Dzantiev, B. B. 2014. Rapid immunochromatographic assay for ofloxacin in animal original foodstuffs using native antisera labeled by colloidal gold. Talanta 119:125-132.
2. Chang, S. H., Chou, T. M., and Kao, C. H. 2016. Activities of the housefly acetylcholinesterase and its susceptibility to chlorpyrifos, chlorpyrifos oxon and propoxur. Formosan Entomol. 36:74-83. (in Chinese with English abstract)
3. Cheng, E. Y., Huang, Y. B., Chiang, M. Y., Hou, Y. H., and Nien, C. C. 2015. The study of pesticide residues control by the combination of AChE screening and chemical analysis: the Taipei model. J. Taiwan Agric. Res. 64:53-63. (in Chinese with English abstract)
4. Chen, W. Y., Chang, C. L., Lee, H. M., Yang, C. H., Liu, F. M., Huang, C. Z., Chiang, H. Z., Lin, Y. B., Hsu, J. H., Maa, H. C., Chen, P. Y., and Chen, C. M. 2017. Monitoring of pesticide residues in agricultural products from markets in Taiwan. Ann. Rept. Food Drug Res. 8:63-75. (in Chinese with English abstract)
5. Chiu, C. S., Kao, C. H., and Cheng, E. Y. 1991. Rapid bioassay of pesticide residues (RBPR) on fruits and vegetables. J. Agric. Res. China 40: 188-203.
6. Engvall, E. and Perlmann, P. 1971. Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. Imm. 8:871-874.
7. Food and drug administration, ministry of health and welfare, executive yuan, Taiwan, ROC. 2017. Method of test for pesticide residues in food-multiresidue analysis (5). Retrieved from https://www.fda.gov.tw/TC/
8. Food and drug administration, ministry of health and welfare, Taiwan, ROC. 2018.

Tolerances for pesticide residues in raw agricultural commodities. Retrieved from https://consumer.fda.gov.tw/Law/PesticideList

9. Hsu, Y. H., Chen, Y. H., Lin, M. C., Liu, Y. C., Chang, H. F., and Shih, D. Y. C. 2014. Survey on pesticide residues in raw materials of traditional chinese medicines (X). Ann. Rept. Food Drug Res. 5:211-223. (in Chinese with English abstract)
10. Hua, X., Yang, J., Wang, L., Fang, Q., Zhang, G., and Liu, F. 2012. Development of an enzyme linked immunosorbent assay and an immunochromatographic assay for detection of organophosphorus pesticides in different agricultural products. PLoS ONE 7:1-11.
11. Qian, G., Wang, L., Wu, Y., Zhang, Q., Sun, Q., Liu, Y., and Liu, F. 2009. A monoclonal antibody-based sensitive enzyme-linked immunosorbent assay (ELISA) for the analysis of the organophosphorous pesticides chlorpyrifos-methyl in real samples. Food Chemistry 117:364-370.
12. USEPA. 2000. The use of data on cholinesterase inhibition for risk assessment of organophosphorus and carbamate pesticides, 53 pp. http://www.epa.gov/pesticides/trac/science/cholin.pdf.
13. Wang, H., Li, G. H., Zhang, Y., Chen, T., and Xue, X. P. 2015. Detection of organphosphorus pesticides residual using lateral flow immune colloidal gold chromatography technology. J. Food Saf. Qual. 6:4409-4415. (in Chinese with English abstract)
14. Watanabe, E., Baba, K., Eun, H., Arao, T., Ishii, Y., Ueji, M., and Endo, S. 2005. Evaluation of a commercial immunoassay for the detection of chlorfenapyr in agricultural samples by comparison with gas chromatography and mass spectrometric detection. J. Chromatogr. A 1074:145-153.
15. Watanabe, E., Baba, K., Eun, H., and Miyake, S. 2007. Application of a commercial immunoassay to the direct determination of insecticide imidacloprid in fruit juices. Food Chem. 102:745-750.
16. Watanabe, E., and Miyake, S. 2013. Quantitative determination of neonicotinoid insecticide thiamethoxam in agricultural samples: a comparative verification between high-performance liquid chromatography and monoclonal antibody-based immunoassay. Food Anal. Methods 6:658-666.

17. Zhang, K., Wu, J., Li, Y., Wu, Y., Huang, T., and Tang, D. 2014. Hollow nanogold microsphere-signalized lateral flow immunodipstick for the sensitive determination of the neurotoxin brevetoxin B. Microchim. Acta 181:1447-1454.

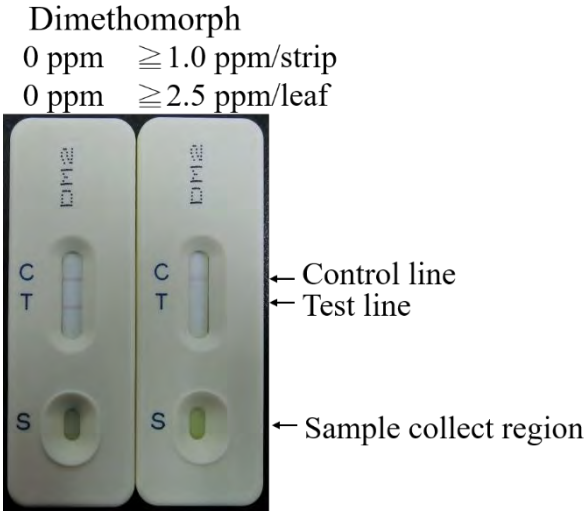

Fig. 1. Dimethomorph in *Brassica rapa* subsp. *chinensis* detected by dimethomorph rapid test strip.

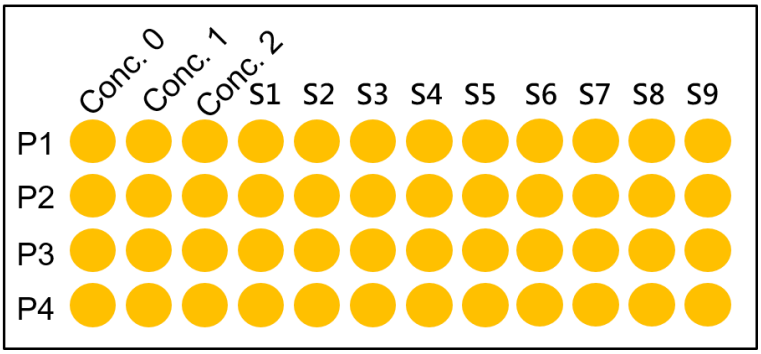

Fig. 2. An ELISA plate is pre-coated separately with 4 pesticides antibodies (P1-P4); and one single plate can estimate the residues of 9 samples (S1-S9) against 4 pesticides simultaneously.